Chapter 5

Proteinase and maturase gene expression in *Lactococcus lactis* is regulated by the transcriptional regulator CodY

Summary

Proteolysis of milk protein (casein) by *Lactococcus lactis* is initiated by the extracellular cell wall-bound proteinase (PrtP). PrtP requires the action of the maturase protein PrtM to become active. Transcription of the divergently oriented *prtP* and *prtM* genes of *L. lactis* strains BGMN1-5 and E8 in response to the peptide concentration in the growth medium was analysed by fusing their intergenic regulatory regions with promoterless α- and β-galactosidase reporter genes. The expression of both *prtP* and *prtM* was shown to be controlled in an identical manner. *prtP* expression was high in medium with a low peptide concentration, and was repressed up to ten–fold by increasing the concentration of medium peptides. Expression of *prtP* was found to be higher than that of *prtM*, an observation that is in contradiction with previously published data. The transcriptional repressor CodY was shown to be responsible for repression of *prtP*/*prtM* transcription in peptide-rich media. Expression of both *prtP* and *prtM* was derepressed in a CodY-deficient *L. lactis* strain. Using electrophoretic mobility shift assays, it was shown that CodY binds to the *prtP*/*prtM* intergenic region.

Introduction

Lactococcus lactis, like other lactic acid bacteria, is a multiple amino acid auxotroph. It has a complex proteolytic system to break down the major milk protein casein into small peptides and free amino acids that are necessary for growth in this medium (Kunji *et al.*, 1996; Christensen *et al.*, 1999). Initial breakdown of casein is carried out by the extracellular cell wall-bound serine proteinase PrtP. Several lactococcal *prtP* genes have been cloned and sequenced (Kok *et al.*, 1985; Kok *et al.*, 1988; de Vos *et al.*, 1989; Kiwaki *et al.*, 1989). Although they are over 98% identical on the amino acid sequence level, the proteinases can have quite different caseinolytic specificities (Visser *et al.*, 1986). For the production of an active proteinase, the product of *prtM*, a gene that is in a back-to-back orientation with *prtP*, is required. The so-called maturase PrtM plays a role as an extracellular chaperone, inducing the pro-proteinase to adopt a conformation in which it is able to autoproteolytically cleave off its pro-region (Kok, 1990; Haandrikman, 1990). Peptides that are produced by the proteinase can be internalised by either one of three different transport systems. Oligopeptides are taken up by Opp, while DtpT and DtpP transport di- and three-peptides respectively (Tynkkynen *et al.*, 1993; Foucaud *et al.*, 1995). Intracellularly, the peptides are further hydrolysed into smaller peptides and amino acids by the action of over 15 different peptidases (Kunji *et al.*, 1996; Christensen *et al.*, 1999).

Proteinase and maturase production is inhibited in peptide-rich medium (e.g. containing casitone, a tryptic digest of casein) in a number of lactococcal strains (Exterkate, 1985; Laan *et al.*, 1993; Marugg *et al.*, 1995; Miladinov *et al.*, 2001). As PrtP expression is not down-regulated in strains that lack the di- and tripeptide transporter DtpT, it was hypothesized that the internal concentration of small (di-tri) peptides, or amino acids derived thereof, are important in the regulation of proteinase production (Marugg *et al.*, 1995). The genetic information for proteinase regulation was shown to be present on a 90-bp subfragment of the *prtP*/*prtM* intergenic region encompassing the transcription start sites of both genes (Marugg *et al.*, 1996). Disruption of an inverted repeat that is present in this region resulted in derepression of the *prtP* and *prtM* promoters in medium with a high peptide concentration. Therefore, it was speculated that this palindromic sequence might be involved in the recognition or binding of a repressor protein, and that specific dipeptides may directly or indirectly act as effectors of this regulator (Marugg *et al.*, 1996).

The expression of genes of other components of the proteolytic system of *L. lactis* is also affected by medium composition. The expression of OppA, DtpT and DtpP is increased when cells are grown in medium with a low peptide concentration (Detmers *et al.*, 1998; Kunji *et al.*, 1996; Foucaud *et al.*, 1995). Moreover, the expression of the peptidases PepX and PepN in *L. lactis* MG1363 was shown to be regulated in a similar way (Meijer *et al.*, 1996). Promoters of *pepC*, *pepN*, *pepO1*, and *pepO2* were also reported to be more active in medium with amino acids than in peptide-rich medium (Guedon *et al.*, 2001a). In the same study, the *prtP* promoter was shown to be subject of a similar regulatory circuit. Recently, it was shown that the genes *pepN*, *pepC* and the *opp-pepO1* operon are regulated by the pleiotropic repressor CodY. The signal controlling CodY-dependent repression was found to be present in dipeptides containing at least one of the branched-chain amino acids (Guedon *et al.*, 2001b).

In this study, the medium-dependent regulation of the *prtP*/*prtM* promoters of the *L. lactis* strains BGMN1-5, E8 and two reference strains, SK11 and Wg2, was studied. In addition, it was shown that CodY can also directly regulate proteinase and maturase expression.

Results

The prtP/prtM intergenic regions of L. lactis BGMN1-5 and E8

Two proteinases with different caseinolytic specificities have been characterised from *L. lactis* strains BGMN1-5 (PI-type) (Gajic *et al*., submitted) and E8 (PI/III-type) (Kok, 1990), respectively. The *prtP* and *prtM* genes of *L. lactis* BGMN1-5 are located on a large plasmid of approx. 80 kb (Gajic *et al.*, 1999). The size of the $PrtP^+$-plasmid of *L. lactis* E8 has not been determined. Using PCR, the *prtP*/*prtM* intergenic regulatory regions of these two strains were amplified. Sequence analysis showed that the lengths of the *prtP*/*prtM* intergenic regions of BGMN1-5 and E8 are identical to that of *L. lactis* SK11, namely 330 bp. In *L. lactis* Wg2, a deletion of 5 bp is present (Fig. 1). Although there are some sequence differences between the three regions the overall homology is very high ($\geq$ 96% identity). The regions are very A/T rich ($\geq$ 75%) and contain several putative –35 and –10 consensus promoter sequences on both strands. As is the case for SK11 and Wg2, the *prtP*/*prtM* intergenic regions of BGMN1-5 and E8 contain an inverted repeat that could possibly form a structure of an estimated ΔG° of –21.4 kcal/mol (-89.5 kJ/mol) and –19.8 kcal/mol (-82.8 kJ/mol), respectively. The ribosome binding sites of *prtP* (GGAGGG) and *prtM* (AAGAG) of BGMN1-5 differ from those present upstream of the corresponding genes of the three other lactococcal strains (GGAGGA and AGGAG, respectively) (Fig. 1).

Determination of the transcription start sites

The transcription initiation sites of *prtP* and *prtM* of *L. lactis* BGMN1-5, E8, and the two reference strains SK11 and Wg2 were determined by primer extension mapping. RNA was isolated from these strains grown in CDM with 0.2% casitone (see below) and annealed with a primer either specific for the 5'-end of *prtP* or that of *prtM*. Our studies confirmed the position of the postulated promoters. Although the lengths of the *prtP*/*prtM* intergenic regions and the –35 and –10 sequences of BGMN1-5 and E8 are identical to that of SK11, the sites at which transcription starts are different in these strains. For both *prtP* and *prtM*, of BGMN1-5 and SK11 two bands are visible on the autoradiogram (Fig. 2). For *prtP*, the upper band, having the highest intensity, corresponds to the transcription initiation site determined previously for *prtP* of SK11 and Wg2 (Vos *et al.*, 1991; van der Vossen *et al.*, 1992) and is therefore designed as +1 (Fig. 1). The lower, less intense band is probably derived from a second, less efficient start site. The *prtP* transcription start site of BGMN1-5 and E8 was found to be a C and a T, respectively, 85 and 84 nucleotides upstream of the proposed AUG start codon.

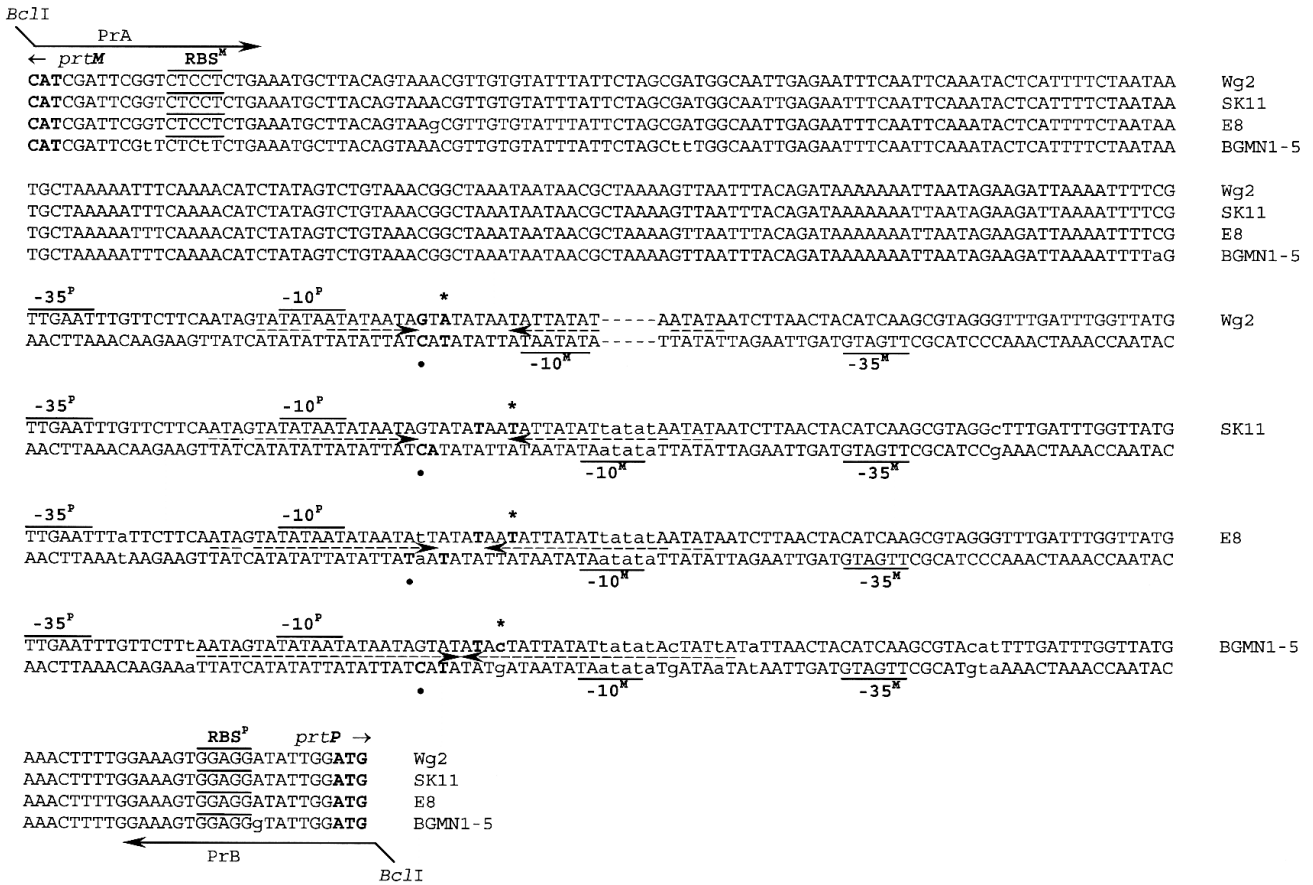

Figure 1. Comparison of the *prtP*/*prtM* promoter regions of *L. lactis* strains Wg2, SK11, E8 and BGMN1-5. The regions between bp 201 and 296 are shown double stranded for each strain, the other (flanking) regions are single stranded. Differences in the nucleotide sequences between the strains are depicted in lower case. *prtP* and *prtM* start codons are indicated in bold and with a small arrow. The deletion in the sequence of *L. lactis* Wg2 is indicated by a dashed line. The major transcription start sites for *prtP* (∗) and *prtM* (•) are indicated above and below the sequence, respectively (see text for details). Minor sites are indicated in bold face. Putative ribosomal binding sites (RBS^P, RBS^M) and -10 (-10^P, -10^M) and -35 (-10^P, -10^M) promoter sequences are overlined. Dashed arrows represent inverted repeats overlapping the promoters. The regions to which the oligonucleotides PrA and PrB anneal are indicated.

The start site in *prtM* of BGMN1-5 and E8 was mapped at 232 bp (G) and 231 bp (A) upstream of the proposed AUG start, respectively (Fig. 1).

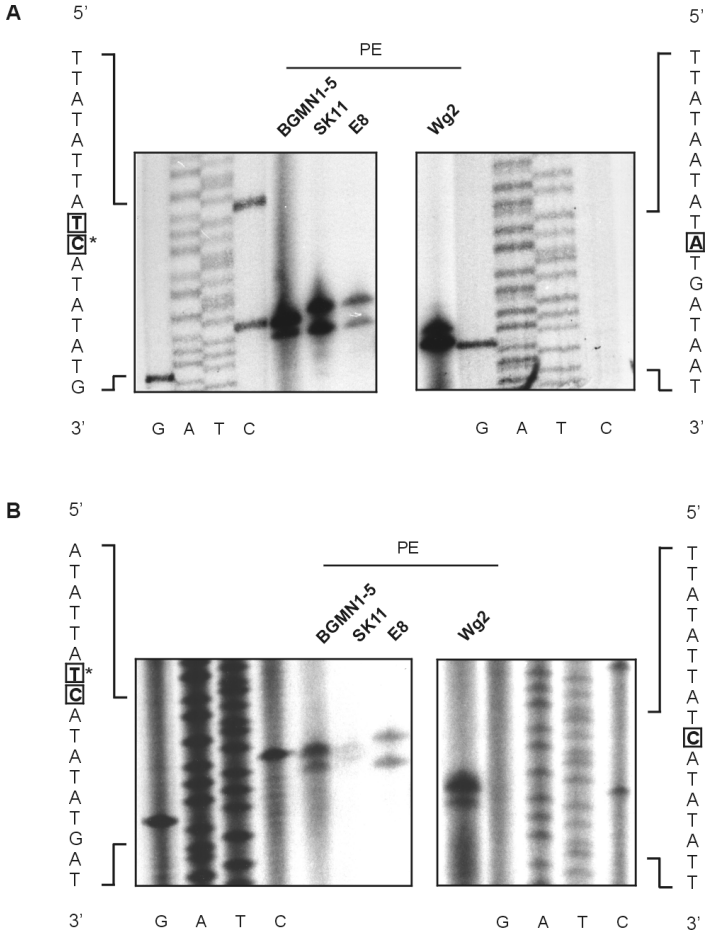

Figure 2. Determination of the transcription start sites of: (**A**) *prtP* and (**B**) *prtM* of *L. lactis* BGMN1-5, SK11, E8 and Wg2 by primer extension mapping. Lanes PE show the extended products using primers PrE (*prtP*) and PrF (*prtM*). Nucleotide sequence ladders, obtained with the corresponding primers and template DNAs of BGMN1-5 and Wg2 were run in parallel. The nucleotide sequences are given in the left- and right-hand margins. The 5' ends of the *prtP* and *prtM* transcripts are boxed. The differences between BGMN1-5 with respect to the other three strains are indicated by asteriks.

Transcriptional regulation of the prtP and prtM promoters

In order to study the regulation of proteinase and maturase production, fragments containing the *prtP*/*prtM* intergenic region of *L. lactis* strains BGMN1-5, E8, SK11 or Wg2, respectively, were cloned in between the promoterless *Escherichia coli* β-galactosidase (*lacZ*) and the *Cyamopsis tetragonoloba* α-galactosidase (*α-gal*) genes of pGKH10. Thus, translational fusions of *prtP* and *prtM* with the two reporter genes were created, the AUG codons of *prtP* or *prtM* serving as a start codon for *lacZ* or *α-gal* (Fig. 3). In the resulting plasmids pGKB11, pGKE11, pGKS11 and pGKH11, *lacZ* is under the control of the *prtP* promoter of BGMN1-5, E8, SK11 and Wg2, respectively. In these plasmids the *prtM* promoter directs the transcription of the *α-gal* gene. In the corresponding plasmids pGKB12, pGKE12, pGKS12 and pGKH12 the respective fragments are present in the opposite orientation.

The plasmids were introduced in the proteinase-deficient *L. lactis* MG1363. For all *prtP* promoters, the highest β-galactosidase activities were observed in the cells grown in CDM containing a low concentration of casitone (0.2%). This pancreatic digest of casein mainly consists of small peptides and amino acids in a ratio of about 4 to 1 (Marrug *et al*., 1995).

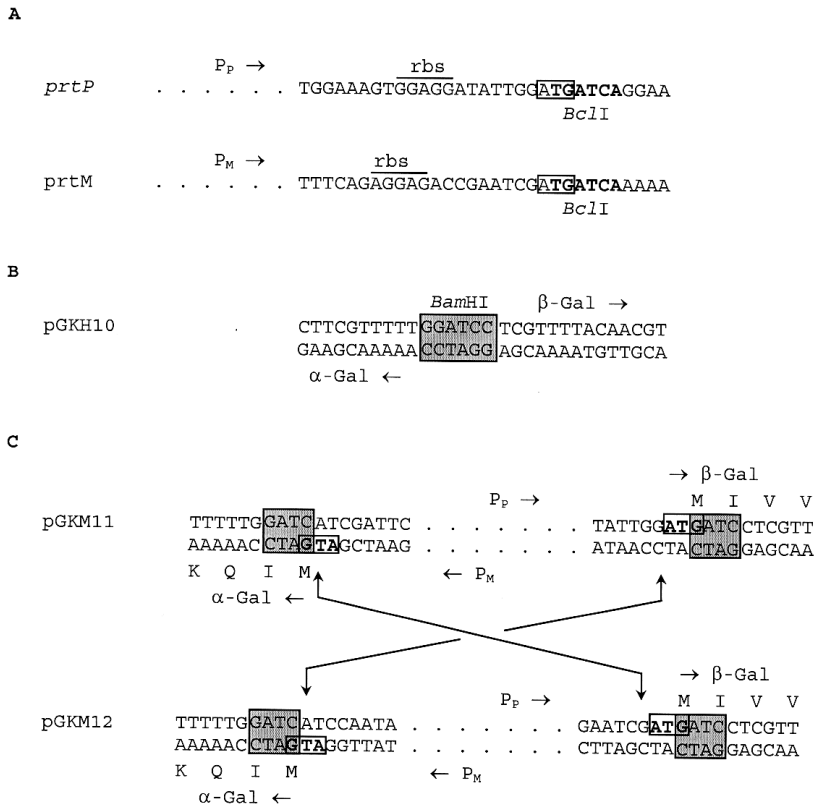

Figure 3. Nucleotide sequence of: (**A**) the DNA regions containing the initiation codons (boxed) of *prtP* and *prtM*. Introduced *Bcl*I site is indicated in bold. Ribosomal binding sites (RBS) are overlined. (**B**) the DNA fragment surrounding the unique *Bam*HI site (boxed) in pGKH10. The orientation of the α- and β-galactosidase genes is indicated. (**C**) DNA regions joined by the *Bam*HI/*Bcl*I fusion in pGKM11 and pGKM12. Direction of transcription of the α- and β-galactosidase genes and the orientation of the expression signals from *prtP* (P_P) and *prtM* (P_M) are indicated. The 4 nucleotides that are duplicated as a consequence of the *Bam*HI/*Bcl*I fusion, are shaded.

The growth rates of the strains decreased dramatically at casitone concentrations lower than 0.1% (data not shown). As in 0.2% casitone growth reached the same optical densities as in rich medium, this casitone concentration was used for the expression studies. Although the β-galactosidase activities differed for the four lactococcal strains tested, a gradual increase in casitone concentration from 0.2% to 2% resulted in a gradual decrease in β-galactosidase activity for all *prtP* promoters (data not shown, and Table 1).

When cells were grown in medium with a high (2%) casitone concentration, β-galactosidase levels were low during growth and were approximately two- to thirteen fold lower than the maximal levels obtained during growth in medium containing 0.2% casitone (Table 1).

Gene expression driven by the *prtM* promoter of strains BGMN1-5, E8, SK11 and Wg2 was analysed in the same way (Fig. 3). As was observed for *prtP*, β-galactosidase activities directed by the *prtM* promoters decreased with increasing concentrations of casitone from 0.2% to 2%. Activity of β-galactosidase decreased approximately four- to ten fold in peptide-rich CDM (2% casitone) compared with that obtained in CDM containing 0.2% casitone (Table 1).

Table 1. β-galactosidase activities of *L. lactis* MG1363 cells carrying pGKH10 derivatives harbouring *prtP*::*lacZ* and *prtM*::*lacZ* fusions[a].

Plasmid		0.2% casitone		2% casitone		
		β-galactosidase activity[b]	P_{prtP}/P_{prtM} ratio[c]	β-galactosidase activity[b]	P_{prtP}/P_{prtM} ratio[c]	Repression[d]
pGKB11	P[e]	15.0 ± 2.0	5	2.5 ± 0.6	8.3	6
pGKB12	M	3.0 ± 0.5		0.3 ± 0.1		10
pGKE11	P	6.5 ± 1.0	1	0.5 ± 0.1	1.25	13
pGKE12	M	6.5 ± 1.1		0.4 ± 0.1		16.25
pGKS1	P	15.0 ± 2.5	2.5	4.0 ± 0.5	4	3.75
pGKS12	M	6.0 ± 1.0		1.0 ± 0.2		6
pGKH11	P	9.0 ± 1.5	1.6	3.5 ± 0.8	3.5	2.57
pGKH12	M	5.5 ± 0.5		1.0 ± 0.2		5.5

[a]Strains were grown in CDM with casitone concentrations as indicated
[b]β-galactosidase activity as determined by fluorescence (calculated as arbitrary fluorescence units divided per time and optical density (AFU x min^{-1} x OD_{595}^{-1}))
[c]Ratio between *prtP* and *prtM* promoter strengths for one strain under identical growth conditions
[d]Ratio between the β-galactosidase activities in CDM with 0.2% casitone and CDM with 2% casitone for one strain
[e]P: *prtP*::*lacZ*; M: *prtM*::*lacZ* fusions

These data indicated that the *prtP* and *prtM* promoters are regulated in a similar way. The expression signals of the *prtP* promoters of *L. lactis* BGMN1-5, SK11 and Wg2 were stronger than those of the corresponding *prtM* promoters in all media tested. The highest β-Gal expression ratio (P_{prtP}::*lacZ*/P_{prtM}::*lacZ*) under the same growth condition was found for the promoters of strain BGMN1-5. No significant differences were observed between both promoters of *L. lactis* E8 (Table 1). Similar data for the β-Gal expression ratios (P_{prtP}::*lacZ*/P_{prtM}::*lacZ*) at low (0.2%) and high (2%) casitone concentrations were obtained when α-galactosidase was measured (data not shown).

The above results are in contradiction with those previously published by Marugg *et al*. (1995; 1996). By using transcriptional fusions of *prtP* or *prtM* with the *E.coli* β-glucuronidase gene, they showed that expression of *prtM* is higher than that of *prtP*. In order to exclude the possibility that a lack of the 5'-end of *prtP* and *prtM* influences the measured values, the promoter region together with the 5'-end of *prtP* and *prtM* (33- and 93-bp, respectively) of strain BGMN1-5 was cloned in two orientations upstream of *lacZ* in pORI13. The constructs were introduced in *L. lactis* LL108. Enzyme expression was analysed by fluorometry during growth of the strains in CDM supplemented with 0.2% or 2% casitone. β-galactosidase production was similar to that observed with the translational fusions presented above. Upon increase of the casitone concentration the β-galactosidase activity decreased and the β-galactosidase activity was higher when the gene was under the control of *prtP* than when it was driven by the *prtM* promoter (data not shown).

Regulation of prtP and prtM is derepressed in a CodY-deficient strain

The pleiotropic repressor CodY was shown to be involved in the regulation of the *pepN* and *pepC* genes and the *opp-pepO1* operon (Guedon *et al.*, 2001b). As these genes are regulated by medium components in a way similar to that of *prtP*, the role of CodY in *prtP* and *prtM* expression was studied. To this end, a CodY-deficient *L. lactis* MG1363 was constructed by replacing the chromosomal copy of *codY*

by a mutated version by means of homologous double cross-over recombination. Plasmids pGKB11 and pGKB12 were introduced in this strain, *L. lactis* MG1363*codYΔ1*, and *prtP* and *prtM* gene expression was analysed in CDM media containing different amounts of casitone, and in peptide-rich M17 medium. In the wild type strain, the β-galactosidase activities of the *prtP*::*lacZ* and *prtM*::*lacZ* fusions were 6- and 8-fold lower in CDM with 2% than in CDM with 0.2% casitone, respectively (Table 2). In the *codY* mutant, β-galactosidase activity in CDM with 2% casitone was less than twofold lower than that in CDM with 0.2% casitone. β-galactosidase activities in M17 were similar to those in CDM with 2% casitone for both MG1363 and MG1363*codYΔ1*. These results show that repression of these two gene fusions by medium peptides was almost fully abolished in the *codY* mutant.

Table 2. Effect of a *codY* mutation on the expression of *prtP*::*lacZ* and *prtM*::*lacZ* fusions in *L. lactis*.

Plasmid		WT β -galactosidase activity[a]			*codYΔ1* β-galactosidase activity[a]		
		0.2	2%	Repression[b]	0.2%	2%	Repression[b]
pGKB11	P[c]	16.0 ± 2.0	2.5 ± 0.5	6.4	25.0 ± 3.2	12 ± 2	2.1
pGKB12	M[c]	2.5 ± 0.4	0.30 ± 0.05	8.3	4.0 ± 0.6	2.5 ± 0.5	1.6

[a]β-galactosidase activity as determined by fluorescence (calculated as arbitrary fluorescence units divided per time and optical density (AFU x min^{-1} x OD_{595}^{-1}))
[b]Ratio between the β-galactosidase activities in CDM with 0.2% and 2% casitone for one strain
[c]P: *prtP*::*lacZ*; M: *prtM*::*lacZ* fusions

CodY binds to the prtP/prtM intergenic region

The *prtP*/*prtM* intergenic region of *L. lactis* BGMN1-5, E8, SK11 and Wg2 each contain an inverted repeat. As it overlaps the –10 sequence of both the *prtP* and *prtM* promoter, it could function as a binding site for a repressor. In order to determine whether CodY is able to bind to this DNA sequence, gel mobility shift assays were performed.

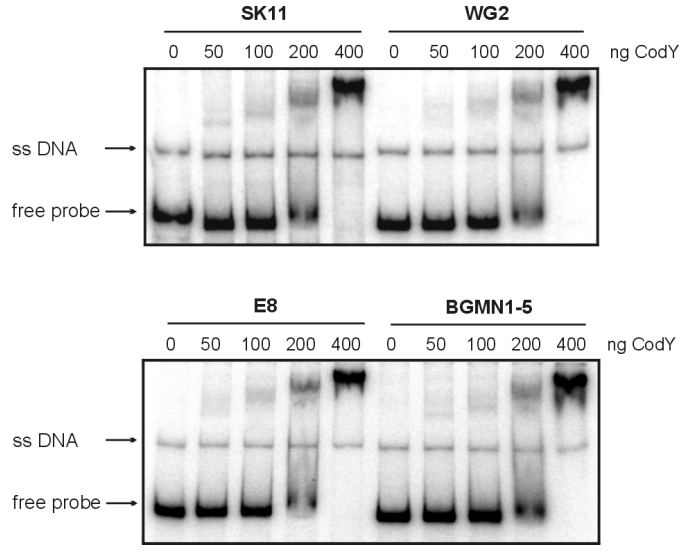

Figure 4. Gel mobility-shift assays performed with the *prtP*/*prtM* promoter regions of *L. lactis* BGMN1-5, E8, SK11 and Wg2. The [γ-^{32}P]-ATP-labelled DNA fragments (3000 cpm) were incubated for 15 min at 30ºC with His6-CodY at concentrations of 0, 50, 100, 200 or 400 ng. Samples were run on a non-denaturing 4% polyacrylamide gel. Free probe and single strand (SS) DNA are indicated.

Histidine(6)-tagged CodY was overexpressed by using the nisin inducible gene expression system (Kuipers *et al.*, 1998). The purified protein was used in a binding study with the 330-bp γ-^{32}P-labelled PCR fragments containing the 330-bp *prtP*/*prtM* intergenic regions of *L. lactis* BGMN1-5, E8, SK11 and Wg2 as probes. Addition of His6-CodY resulted in a markedly lower electrophoretic mobility of all four double stranded PCR products on a polyacrylamide gel as compared to the situation in which CodY was not added (Fig. 4). Moreover, multiple shifted bands are present suggesting that CodY might act as a multimer. These results indicate that CodY binds to the *prtP*/*prtM* intergenic regions of all four lactococcal strains tested.

Discussion

Nucleotide sequence analysis of the intergenic regions encompassing the divergently oriented *prtP* and *prtM* promoters of different *L. lactis* strains showed that, although base-pair substitutions are present, the length of this region in *L. lactis* BGMN1-5 and E8 is identical to that in SK11 (Vos *et al.*, 1989). Transcription initiation sites for the *prtP* and *prtM* genes of BGMN1-5 and E8 were mapped and found to be in close proximity to each other, as is the case for *prtP*/*prtM* genes of SK11 and Wg2 (Vos *et al.*, 1989; van der Vossen *et al.*, 1992).

By using transcriptional and translational fusions of the *prtP* and *prtM* promoters of *L. lactis* BGMN1-5, E8, SK11 and Wg2 with promoterless reporter genes, it was shown that the *prtP* expression level was higher than that of *prtM* in all strains except E8. This observation is in contradiction with results previously published by Marugg *et al*. (1995; 1996). In their studies these authors used transcriptional fusions of the *prtP* and *prtM* promoters from *L. lactis* SK11 with a promotorless β-glucuronidase gene (*gusA*). As judged from the plasmid pNZ544 and pNZ554 construction (Marugg *et al.*, 1995) and the published nucleotide sequence of the 0.35-kb *Sal*I-*Eco*RI fragment containing the *prtP* and *prtM* promoters (Marugg *et al.*, 1996), the AUG start codon of *prtM* is not present in pNZ554: by cloning Klenow-treated *Cla*I fragment, carrying the *prtP*/*prtM* promoters, into the Klenow-treated *Sal*I site of the vector, the AUG has been replaced by CGA, while the ribosome binding site of *prtM* was present. The start codon and the ribosome binding site of *prtP* were present in pNZ544. Nevertheless, in both gene fusions translation initiates at the AUG of *gusA*, as stop codons are present in the region upstream of the ribosome binding site of *gusA*. The differences between the two fusions could account for the observed P_{prtP}::*gusA*/P_{prtM}::*gusA* expression ratio in their study.

As is the case for the expression of peptide transport systems of *L. lactis*, namely DtpP (Foucaud *et al.*, 1995), DtpT (Kunji *et al.*, 1996) and OppA (Detmers *et al.*, 1998) and several peptidases e.g. PepN, PepC, PepO1 and PepO2 (Guedon *et al.*, 2001b; 2001a), PrtP and PrtM were also shown to be repressed in cells grown in the presence of peptide-rich sources such as casein hydrolysate, casitone and casamino acids (Marugg *et al.*, 1995; 1996; Guedon *et al.*, 2001a). For the above-mentioned enzymes and proteins it was shown that their production is negatively regulated by the dipeptides Leu-Pro and Pro-Leu. It was suggested that they might form (part of) a signal that mediates the regulation on a rich nitrogen source (Marugg *et al.*, 1995; Guedon *et al.*, 2001a; 2001b). Guedon *et al*. (2001b) showed that the expression of *pepN*, *pepC* and *opp-pepO1* is regulated by CodY and that branched-chain amino acids serve as a signal for CodY activity. As almost all products involved in casein degradation are regulated in a similar medium-dependent way, the involvement of a common regulator was suggested. Here, we show that the expression of the *prtP* and *prtM* genes is approximately 10-fold repressed by CodY in media with a high concentration of peptides.

In the *prt* regulatory region of all four strains, a sequence with dyad symmetry is present which could form a stem-loop structure with a free energy (ΔG°) varying from –10.2 to –21.4 kcal/mol between

the strains. Marugg *et al*. (1996) hypothesized that specific sequences within this repeat structure may be involved in regulation of expression of both *prt* genes, since removal of half of this repeat in SK11 resulted in an almost constitutive expression of the *prtP* promoter. The dyad symmetry in *L. lactis* BGMN1-5 has the highest estimated ΔG° (–21.4 kcal/mol; -89.5 kJ/mol), which could explain that the regulation of *prt* gene expression is most stringent in this strain. As was suggested before (Marugg *et al.*, 1996), the palindromic sequence in the intergenic region between *prtP* and *prtM* could be involved in the recognition and/or binding of a repressor protein. By using gel retardation experiments, we confirmed that CodY binds to the *prtP*/*prtM* promoter region. Multiple bands are observed, suggesting that CodY might act as a multimer. Multiple shifted bands were also observed when the *dpp* (dipeptide permease) promoter region of *Bacillus subtilis* was used in gel mobility shift assays with the *B. subtilis* CodY protein (Serror *et al.*, 1996b). *B. subtilis* CodY was also shown to bind to the *srfA* and *comK* promoters of this species, but no consensus sequence to which it binds could be deduced from the CodY protected regions (Serror *et al.*, 1996a).

Experimental procedures

Bacterial strains, plasmids and media

The strains and plasmids used in this study are listed in Table 3. *L. lactis* was grown at 30°C in M17 broth (Terzaghi *et al.*, 1975) or on M17 agar plates containing 1.5% (wt/vol) agar. Chemically defined medium (CDM) (Mierau *et al.*, 1994) containing 0.2% or 2% (wt/vol) casitone (Difco Laboratories, Detroid, Mich. USA) was used for the expression studies. All media contained 0.5% (wt/vol) glucose, while 5 μg/ml chloramphenicol (Sigma-Aldrich, St. Louis, Missouri, USA) or 5 μg/ml erythromycin (Roche Molecular Biochemicals, Mannheim, Germany) were added when necessary. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma-Aldrich) was used at a concentration of 1 mM.

DNA techniques and transformation

Molecular cloning techniques were performed essentially as described by Sambrook *et al.* (1989). Restriction enzymes, Klenow enzyme, T4 DNA ligase and Expand^{TM} High Fidelity DNA polymerase were used according to the instructions of the supplier (Roche Molecular Biochemicals). Synthetic oligo deoxyribonucleotides were obtained from Life Technologies B.V. (Breda, the Netherlands). PCR products were purified with the High pure PCR product purification kit (Roche Molecular Biochemicals). For nucleotide sequence analysis the dideoxy chain termination method (Sanger *et al.*, 1977) was used with [α-^{35}S]-dATP (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) and the T7 sequencing kit (Amersham Pharmacia Biotech). *L. lactis* was transformed by electroporation using a gene pulser (Bio Rad Laboratories, Richmond, Calif.) as described by Leenhouts and Venema (1993).

Plasmid constructions

The *prtP/prtM* promoter region of *L. lactis* BGMN1-5 was amplified by PCR with the oligonucleotides PrA (5'-CTCGTGATCATCGATTCGTTCTCTTCTG) and PrB (5'-CAGGTGATCATCCAATACCCTCCACTTTCC). Oligonucleotides PrD (5'-CAGGTGATCATCCAATATCCTCCACTTTCC) and PrC (5'-CTCGTGATCATCGATTCGGTCTCCTCTG) were used for the amplification of the *prtP*/*prtM* intergenic regions of *L. lactis* E8, SK11 and Wg2. Purified PCR products were digested with *Bcl*I (restriction site is underlined in the oligonucleotide sequence), and ligated into the *Bam*HI site of pGKH10 (Haandrikman, 1990), upstream of the promoterless α-galactosidase (α*gal*) and β-galactosidase (*lacZ*) genes. All constructs were made using *L. lactis* MG1363 as the cloning host. The *prtP* and *prtM* promoters of BGMN1-5 were each transcriptionally fused to *lacZ* in pORI13. To this end, the PCR product obtained on BGMN1-5 template DNA with PrE (5'-ACCAGCCAGACTAAACTGAC; annealing to the 5'-end of *prtM*) and PrF (5'-GGCTAACAAGATCGATAGCCC; annealing to the 5'-end of *prtP*) as oligonucleotides, was cloned into the *Sma*I site of pORI13. The ligation mixtures were used to transform *L. lactis* LL108. This strain carries the *repA* gene on the chromosome, thereby allowing pORI13 derivatives to replicate. The orientation of the inserts was confirmed by nucleotide sequencing.

Overproduction and purification of His$_6$-CodY

Chromosomaly located *codY* of *L. lactis* MG1363 was amplified by PCR with oligonucleotides HC-5 (5'-CTAGACCA CCATGGGG*CATCACCATCACCATCAC*GTGGCTACATTACTTGAAAAAACACG), introducing the underlined *Nco*I

restriction enzyme site upstream of the hexa-histidine tag (italic) and HC-6 (5'-CTAGTCTAGATT*TAG*AAATTACGT CCAGCAAGTTTATC), introducing the underlined *Xba*I restriction enzyme site downstream of the stop codon (italic) of *codY*. The purified 833-bp PCR product was digested with *Nco*I and *Xba*I and ligated into the corresponding sites in pNZ8048, resulting in pNH6CodY. This plasmid was introduced in *L. lactis* NZ9000 to enable nisin induction of P_{nisA} upstream of *his6-cody*, as described by de Ruyter *et al.* (1996). Subsequently, His6-CodY was isolated by affinity chromatography in an FPLC procedure (Amersham Pharmacia Biotech) using Ni-NTA agarose (Qiagen GmbH, Hilden, Germany).

Table 3. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
L. lactis subsp. *cremoris*		
MG1363	Lac^-; Prt^- ; Plasmid–free derivative of NCDO712	Gasson, 1983
MG1363*codYΔI*	MG1363 derivative with chromosomal deletion in *codY*	This work
NZ9000	MG1363 *pepN*::*nisRK*	Kuipers *et al.*, 1998
NZ9700	Nisin producing transconjugant containing the nisin-sucrose transposon Tn*5276*	Kuipers *et al.*, 1993
LL108	Cm^r, MG1363 derivative containing pWV01 *repA* gene in the chromosome	Leenhouts *et al.*, 1998
SK11	Lac^+; Prt^+; harbours, a.o., proteinase plasmid pSK111	de Vos *et al.*, 1984
Wg2	Prt^+; harbours, a.o., proteinase plasmid pWV05	Otto *et al.*, 1982
E8	Lac^+; Prt^+	Kok, 1990
L. lactis subsp. *lactis*		
BGMN1-5	Wild type strain, $PrtP^+$, $Bac501^+$, $Bac513^+$	Gajic *et al.*, 1999
Plasmids		
pNZ8048	Cm^r, inducible expression vector carrying P_{nisA}	Kuipers *et al.*, 1998
pNH6CodY	*his6-codY* of *L. lactis* MG1363 behind P_{nisA}	This work
pORI280	Em^r , *ori*$^+$ of pWV01, replicates only in strains carrying *repA in trans*	Leenhouts *et al.*, 1996
pORI*ΔcodY*	pORI280 derivative containing 423 bp deletion in *codY*	This work
pVE6007	Cm^r, Ts replication derivative of pWV01	Maguin *et al.*, 1992
pGKH10	Em^r, Cm^r, contains promoterless genes for α- and β-gal	Haandrikman, 1990
pGKH11	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtM} and P_{prtP} of Wg2, respectively	Haandrikman, 1990
pGKH12	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtP} and P_{prtM} of Wg2, respectively	Haandrikman, 1990
pGKB11	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtM} and P_{prtP} of BGMN1-5, respectively	This work
pGKB12	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtP} and P_{prtM} of BGMN1-5, respectively	This work
pGKE11	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtM} and P_{prtP} of E8, respectively	This work
pGKE12	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtP} and P_{prtM} of E8, respectively	This work
pGKS11	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtM} and P_{prtP} of SK11, respectively	This work
pGKS12	Em^r, Cm^r, contains genes for α-gal and β-gal, controlled by P_{prtP} and P_{prtM} of SK11, respectively	This work
pORI13	Em^r, Cm^r, contains promoterless *lacZ*, *repA*$^-$	Sanders *et al.*, 1998
pORIB11	Em^r, Cm^r, pORI13 derivative carrying *lacZ* gene fused to *prtP* promoter of BGMN1-5	This work
pORIB12	Em^r, Cm^r, pORI13 derivative carrying *lacZ* gene fused to *prtM* promoter of BGMN1-5	This work

Em^r, Cm^r Ap^r, resistance to erythromycin, chloramphenicol and ampicilin, respectively. P_{nisA}-inducible *nisA* promoter, P_{prtP} – *prtP* promoter, P_{prtM} - *prtM* promoter, α-gal – α-galactosidase, β-gal – β-galactosidase.

Construction of a codY deletion strain

A 1400 bp *Eco*RI/*Hin*dIII chromosomal fragment of *L. lactis* MG1363, containing *codY*, was subcloned in pUC19. The resulting plasmid was digested with *Sna*BI and subsequently selfligated. In this way, 423 bp were deleted from *codY*. The oligonucleotides cod280A (5'-GGGAATTCGGATTGTCTATCTGCCTCG) and cod280B (5'-GGGGGATCCAGATCTGACC ATGATTACGCCAAGCTT) were used to amplify the Δ*codY*-containing fragment. PCR product was digested with *Eco*RI/*Bam*HI (restriction sites are underlined in the oligonucleotide sequence) and ligated into corresponding sites in pORI280. The resulting plasmid, pORIΔ*codY*, was introduced together with pVE6007 into *L.lactis* MG1363. As this strain does not contain the *repA* gene, selection for growth in the presence of erythromycin and increased temperature (37°C) forces pORIΔ*codY* to integrate into the chromosome by homologous recombination. A number of integrants were subsequently grown for about 30 generations under nonselective conditions allowing a second recombination event to occur, which results in either the deletion or the wild-type gene *codY*. The Δ*CodY* mutation was confirmed by PCR and Southern hybridisation experiments.

α- and β-galactosidase activity assays

The activities of α- and β-galactosidase were measured during growth of *L. lactis* in a 96 well microtiter plate (Greiner Bio-One B.V., Alphen, the Netherlands) using the GENios microtiterplate reader and Magelan software (Tecan, Grödig, Austria). β-galactosidase activity was measured by determining the conversion rate of T657 substrate (trifluoromethyl umbelliferyl-β-D-galactopiranoside; Molecular probes, Leiden, the Netherlands) into T659 fluorescent product. α-galactosidase activity was measured by using 4-methylumbelliferyl-α-D-galactopyranoside (Fluka Chemie, Zwijndrecht, the Netherlands). Fluorescence was followed using excitation and emission wavelengths of 360 and 535 nm, respectively. Culture optical densities (OD) were measured at 595 nm. Specific α- and β-galactosidase activities (mid exponential growth phase; OD~0.7) were calculated as arbitrary fluorescence units divided per time and optical absorbance (AFU x min^{-1} x $OD_{595}{}^{-1}$).

Primer extension analysis

RNA was isolated from exponentially growing *L. lactis* cells as described by van Asseldonk *et al*. (1993). Synthesis of cDNA was performed using SUPERSCRIPT transcriptase (Life Technologies). mRNA (3.5 μg) was reverse transcribed using 25 ng of the synthetic oligonucleotides PrE or PrF and 0.25 mM dATP, dGTP, dTTP, and 2μM [α-^{32}P]dCTP (Amersham Pharmacia Biotech). Reactions were performed as described by Sanders *et al*. (1998).

Gel retardation

Gel retardation experiments were carried out essentially as described by Ebbole and Zalkin (1989). Oligonucleotides PrA and PrB were used to amplify the *prtP/prtM* promoter region of *L. lactis* BGMN1-5, while PrC and PrD were employed to amplify corresponding regions of *L. lactis* E8, SK11 and Wg2. Purified PCR products (2 μg) were end-labelled with polynucleotide kinase (Amersham Pharmacia Biotech) for 1 h at 37°C using 30 μCi [γ-^{32}P]-ATP (Amersham Pharmacia Biotech). Reactions were stopped by incubating the mixtures for 10 min at 70°C. Binding studies were carried out in 20 μl reaction volumes containing 20 mM Tris-HCl (pH 8.0), 8.7 % (v/v) glycerol, 1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 0.5 mM DTT, labelled DNA fragment (3000 cpm), and purified His6-CodY protein (50-400 ng). After incubation for 15 min at 30°C, samples were loaded onto a 4% polyacrylamide gel. Electrophoresis was performed in the Protean II Minigel System (Bio Rad) using a gradient of TAE (Sambrook *et al.*, 1989) buffer (0.5x to 2x) at 150 V for 1.5 h. Gels were dried and used for autoradiography at –80°C using Kodak XAR-5 films and intensifying screens.

References

References are listed in Chapter 9